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# THE ISOLATION OF NON-KERATIN PROTEIN FILAMENTS FROM INNER ROOT SHEATH CELLS OF THE HAIR FOLLICLE*

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## ABSTRACT

Protein filaments obtained from the cells of the inner root sheath layers of the guinea pig hair follicle have been isolated and characterized. They are hollow tubes approximately 80 Å in diameter and are of indeterminate length. The protein of the filaments is unique in that it contains the amino acid citrulline and its amino acid composition is very similar to that of the total protein obtained from the inner root sheath cells as soluble polypeptides by digestion with crystalline trypsin.

The intracellular filaments of the inner root sheath cells are chemically and structurally quite distinct from the keratin microfibrils that are present in the neighboring cortical cells of the follicle. It is suggested that the filaments play a role in the development of cell shape in the hair follicle in a manner analogous to that which is accepted for microtubules in many types of cells.

The proteins contained in the mature inner root sheath cells of hair follicles and the medullary cells of hair fibres have been shown to contain substantial amounts of the amino acid citrulline (1–7). Since the protein contained in these tissues is highly resistant to dissolution by normal protein solvents (2), chemical studies have been performed on soluble polypeptides derived from the tissues by digestion with crystalline trypsin or pepsin. Limited sequence analyses of polypeptides derived from the proteins of these and a related tissue (the medulla of porcupine quills) indicates that the citrulline is chemically bound in the proteins by peptide linkages (3, 7).

Previous studies have indicated that the protein of the inner root sheath is composed of filaments of the $\alpha$-type (5, 6), oriented in the cell in the direction of fibre growth, while the protein of the medulla is not frankly filamentous (6). In this paper we show that the protein of the inner root sheath can be isolated in the form of morphologically distinct filaments and describe their properties.

## MATERIALS AND METHODS

The hair follicles of male albino guinea pigs aged one to three weeks were exposed by the wax-sheet procedure (6, 8), removed with animal clippers and suspended in a buffer of 10 mM tris-chloride pH 7.4 containing 10 mM KCl.

*Isolation of mature inner root sheath tissue.* The published method (5, 6) for the preparation of inner root sheaths was unsatisfactory due to the presence of hair and denatured insoluble protein. Consequently, follicles were dispersed by gentle agitation in a buffer of 8 M urea, 10 mM tris-chloride pH 7.4, 25 mM 2-mercaptoethanol at 4° for 5 min. The soluble prekeratin and other cytoplasmic proteins were alkylated at pH 9.0 by addition of solid iodoacetic acid to 50 mM until —SH negative and centrifuged at 38,000 g for 15 min. The pellet containing the mature inner root sheaths, keratinized hair and cellular debris was washed free of the solubilized proteins and urea by centrifugation at 500 g in 10 mM tris-chloride buffer pH 7.4, resuspended in 5 ml of 10% sucrose in this buffer and layered onto a 24 ml discontinuous sucrose gradient in a 30 ml cellulose-nitrate tube. The gradient employed was composed of 6 ml layers of 70, 60, 50 and 20% sucrose in the same buffer and was centrifuged at 22,500 rpm for 30 min. in a Spinco SW25.1 rotor. Only inner root sheaths banded at the 50–60% sucrose interface. The denser hair fibres banded at the 60–70% sucrose interface and the other cytoplasmic debris remained at higher levels on the gradient. The purified sheaths were examined by light microscopy using phase-contrast and polarized-light optics and were observed to be free of other particles. The sheaths were recovered and washed by centrifugation to remove sucrose.

*Preparation of filaments.* Filaments could be released from inner root sheaths isolated by the procedure described above. The most satisfactory method for release was limited digestion with a 0.1% solution of a proteolytic enzyme in the tris-

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chloride-KCl buffer. Four different enzymes were tested: crystalline trypsin (Sigma, Type III, 2-times crystallized), α-chymotrypsin (Worthington, 3-times crystallized), pronase (Calbiochem, B-grade) and Difco trypsin (Difco Labs., 1:250 preparation). The enzyme solutions were clarified by centrifugation at 50,000 rpm in a Spinco 50.1 rotor for 2 hr immediately before use. Digestions were performed in a "filtration apparatus" similar to that described by Kawiak *et al.* (9) for times varying up to 15 min at 20°. At the completion of the digestion the cell suspensions were chilled to 0° and the cells harvested and washed by centrifugation in the buffer at 500 g to remove enzyme. Filaments were then released from the cells by homogenization in the tris-chloride-KCl buffer in a close-fitting Dounce homogenizer (Kontes glass; clearance approximately 0.07 mm). The homogenates were centrifuged at 4,000 g for 5 min to remove cellular debris. The resultant supernates contained the inner root sheath protein filaments and were retained for further studies.

The same procedure could be applied to intact follicles thus avoiding the pre-isolation of the inner root sheaths. In these circumstances the cells of the follicle bulbs were completely digested within about 10 min; the surrounding inner root sheaths had become detached from the follicles and were disrupted into single cells which could then be readily separated in the filtration apparatus.

*Trypsin digestion of inner root sheaths to polypeptides.* The standard method in protein chemistry for the enzymic cleavage of proteins to polypeptides is to use purified proteolytic enzymes of known specificity. Accordingly, the procedure for the release of the *total* protein of the inner root sheath tissue was by digestion with a pure, crystalline proteolytic enzyme. As in earlier sequence studies (7), the digestions were performed using crystalline trypsin (Mann, minimal chymotrypsin content) at 37° in 10 mM $NH_4HCO_3$ (pH 8.3) using an enzyme:tissue ratio of 1:100. The reaction was terminated after 3 hr by freeze-drying the supernate obtained by centrifugation for 5 min at 4,000 g.

*Amino acid analysis.* Samples for amino acid analysis were hydrolyzed for 28 hr in constant-boiling HCl at 110°, freed of HCl by evaporation and analyzed in a Technicon amino acid analyser. Filament preparations were dialyzed for 4 hr against water and then freeze-dried before hydrolysis.

*Electron microscopy.* Specimens for electron microscopy were examined in a Siemens Elmiskop I electron microscope. Homogenate preparations were examined after negative-staining with 2% uranyl acetate. Freshly depilated guinea pig hair follicles were fixed in 2% glutaraldehyde, post-fixed in 1% osmic acid, dehydrated in acetone and embedded in araldite by standard procedures. Sections were stained on the grid for 90 min with 1% potassium permanganate.

## RESULTS

*Fine structure of filaments* in situ. A transverse cross-section through the inner root sheath layer of a guinea pig hair follicle is shown in Fig. 1a at a stage when the Henle layer of the sheath has become a hardened rigid structure. Filaments appear as hollow tubes about 80 Å in diameter. Fig. 1b shows a transverse cross-section through the cortical region of the follicle at the same level. Keratin microfibrils approximately 80 Å in diameter can be seen. They are clearly different in their staining properties. Their "cores" and the interfilamentous regions (matrix) are more densely stained than the inner root sheath filaments.

*Isolation of filaments.* In earlier studies (2) attempts to release the fibrous protein material of the inner root sheath by a variety of techniques were not successful. However, it had been noted that during proteolytic digestion of the inner root sheaths (to release the protein as soluble polypeptides), the sheaths were initially disrupted into single highly-birefringent cells, the fibrous contents of which were removed only after longer digestion. We have found that homogenization of the cells produced during the early stages of this digestion releases the protein filaments.

Homogenization of sheaths in buffer before enzyme treatment did not produce filaments, but instead, large membrane-bound clumps of fibrous material. Moreover, homogenates of intact hair follicles prepared in the tris-chloride buffer did not show the presence of filaments. Of the proteolytic enzymes tested, the method employing digestion with Difco trypsin, for approximately 15 min at 20° on intact follicles or purified inner root sheaths gave the highest yields of filaments per unit weight of starting material.

A typical preparation of filaments negatively-stained by uranyl acetate is shown in Fig. 2a. These filaments were prepared from whole guinea pig hair follicle tissue by the method described. The filaments are approximately 80 Å in width, several microns in length and appear to have a distinct "core" throughout their length (Fig. 2b).

It was determined by electron microscopy using a variety of negative-staining techniques, that such preparations of filaments were almost completely devoid of other cytoplasmic particles

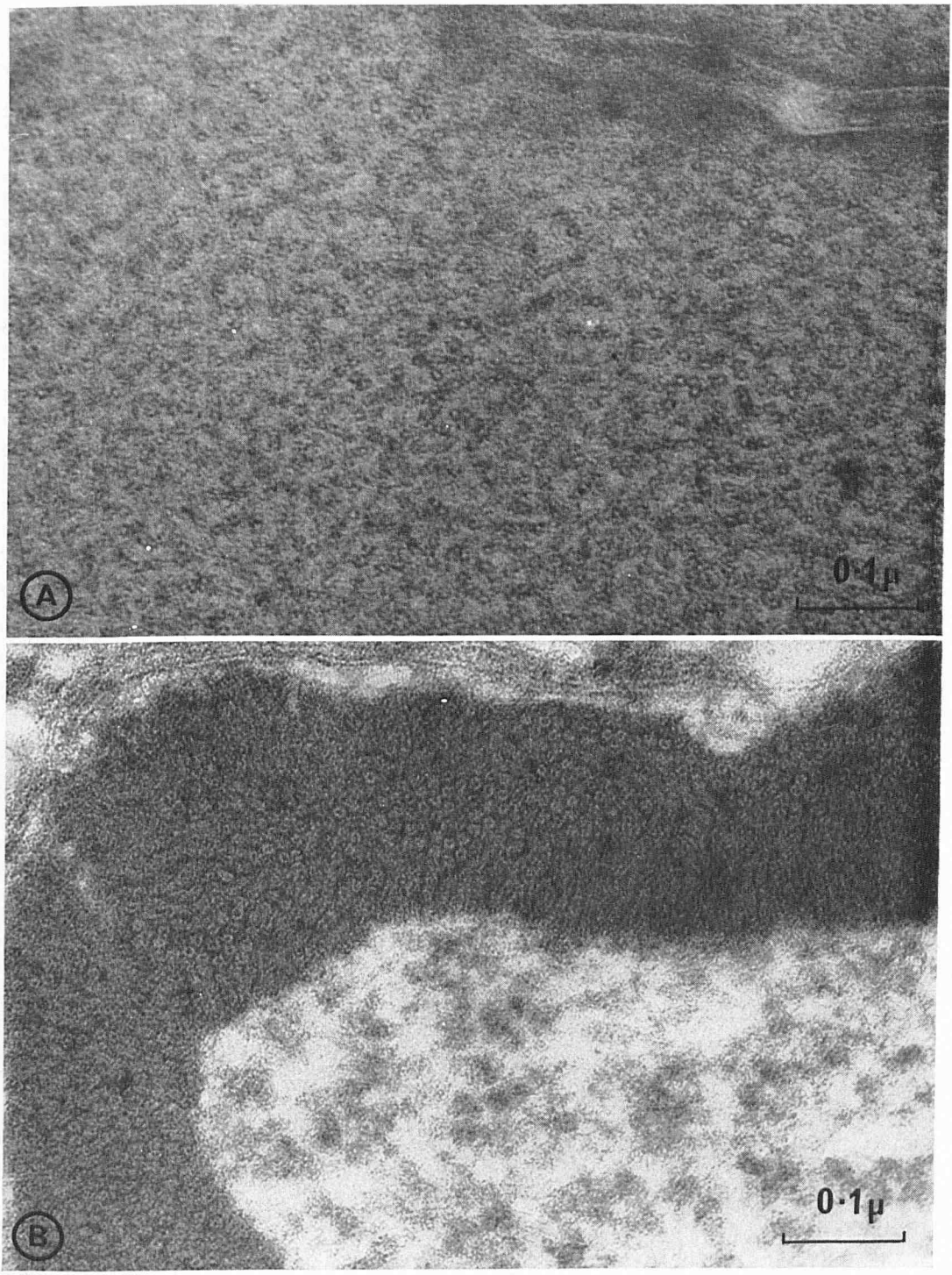


FIG. 1. Transverse cross-section through a guinea pig hair follicle. (a) Cross-section through a cell of the Henle layer of the inner root sheath showing the hollow tubular structure of the filaments. The filaments are approximately 80 A in diameter. The inter-filamentous regions are of very low electron density. (b) Cross-section through the cortical region of the follicle at the same level. Each microfibril is approximately 80 A in diameter and has a densely-stained core and the microfibrils are surrounded by a densely-staining matrix. Specimens prepared as described in Materials and Methods. × 180,000.

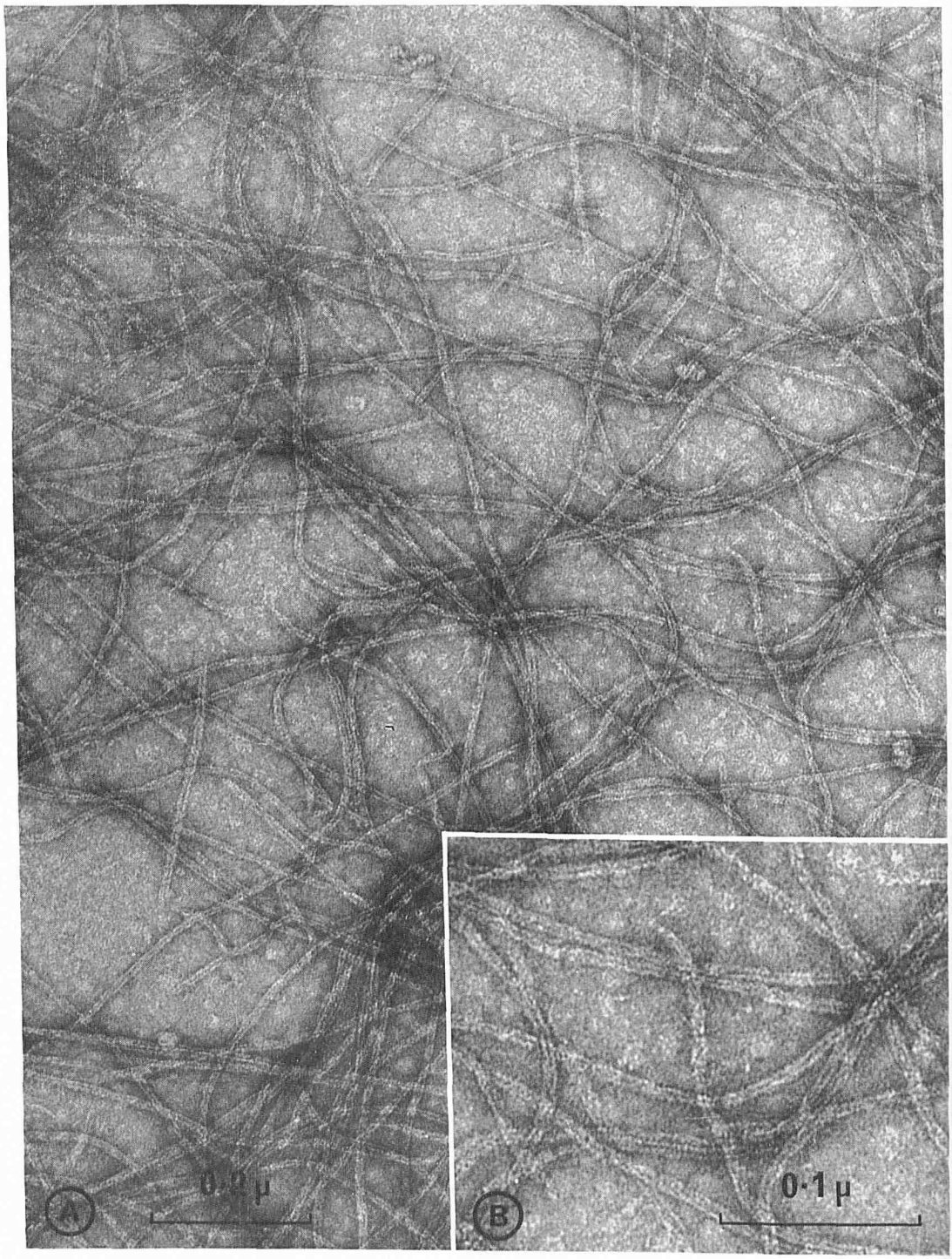


FIG. 2. Inner root sheath filaments prepared from whole guinea pig follicle tissue by digestion with Difco trypsin for 15 min at 20°. The majority of the filaments shown here are about 80 A in diameter and are more than 1 micron long. They show a dense core throughout their length which appears to constitute one third of their diameter. Negatively-stained with 2% uranyl acetate. (a) × 120,000. (b) × 210,000.

and were considered to be sufficiently pure for further studies.

*Amino acid analyses.* It can be seen from the Table that the amino acid composition of the inner root sheath filament preparations and the low sulfur keratin proteins (H-SCMK-A) extracted from guinea pig hair are completely different (columns 1–3 cf column 4). Comparison of the inner root sheath filament preparations with the low sulfur keratin proteins, instead of the total keratin proteins of hair (H-SCMK), is justified as this fraction is thought to derive from the microfibrillar moiety of the original hair fibre (10). Thus the inner root sheath filaments are chemically as well as structurally unlike those of keratin.

The analyses of the filaments are quite similar to the analyses of the tryptic polypeptides derived from the *total* proteins of the inner root sheath. There are, however, four amino acids which vary significantly; in the filaments the content of citrulline is higher and the contents of tyrosine, phenylalanine and lysine are lower than those in the tryptic peptides of whole inner root sheaths. This suggests the presence in whole inner root sheaths of other protein species that do not contain citrulline and which are absent from the filament preparations.

TABLE

*Amino acid analyses of the protein samples*

(All values are expressed as moles percent and represent the average of two experiments)

| Amino acid | Filaments isolated from inner root sheaths | Tryptic polypeptides derived from inner root sheaths | H-SCMK-A* |
|---|---|---|---|
| SCM-cysteine | 0.0 | 0.0 | 5.3 |
| Aspartic acid | 10.2 | 9.4 | 8.7 |
| Threonine | 3.2 | 3.0 | 4.2 |
| Serine | 6.5 | 5.8 | 6.4 |
| Glutamic acid | 24.0 | 22.5 | 16.7 |
| Proline | 4.1 | 3.5 | 3.5 |
| Citrulline† | 4.1 | 3.2 | 0.0 |
| Glycine | 7.7 | 7.1 | 4.4 |
| Alanine | 6.6 | 6.2 | 6.6 |
| Valine | 4.8 | 4.8 | 5.8 |
| Half-cystine | 0.7 | 0.7 | 0.0 |
| Methionine | 2.3 | 2.1 | 0.4 |
| Isoleucine | 3.4 | 3.2 | 3.5 |
| Leucine | 9.8 | 9.1 | 10.0 |
| Tyrosine | 1.9 | 2.7 | 1.9 |
| Phenylalanine | 1.7 | 3.1 | 1.5 |
| Ornithine | 1.4 | 1.0 | 0.0 |
| Lysine | 4.3 | 8.8 | 3.7 |
| Histidine | 1.4 | 1.4 | 0.9 |
| Arginine | 3.6 | 3.7 | 7.0 |
| % Recovery of protein material hydrolyzed as amino acid | 74 | 78 | 93 |

* From unpublished studies. The protein was extracted from guinea pig hair (H) by reduction and then alkylation with iodoacetic acid to give the S-carboxymethyl kerateine (SCMK) derivative in which the half-cystine residues have been converted to SCM-cysteine residues. Fraction A is prepared by precipitation at pH 5.0. This kerateine fraction (H-SCMK-A) is lower in SCM-cysteine content than the total hair keratin proteins (H-SCMK) and is believed to derive from the microfibrils of the original hair fibre (10).

† During acid hydrolysis citrulline undergoes partial decomposition to ornithine. The citrulline value given is the sum of the citrulline remaining after 28 hr and the ornithine produced during this time.

## DISCUSSION

*General properties.* It has been recognized for some time from electron microscope studies that the cells of the mature inner root sheath mainly contain oriented filaments about 80 Å in diameter (5, 6). Previous studies on the total proteins derived from the tissue have shown the presence of the amino acid citrulline (1–7). However, it was not known whether the citrulline was located in the filaments or in some other protein present in the inner root sheath cells. Attempts to isolate the fibrous protein or an intact protein species had not been successful. Consequently, previous chemical studies were performed on soluble polypeptides derived from the tissue which was the only known means of removing the protein content from the cells. In contrast, the release of the filaments from the inner root sheath cells as described in the present work has been achieved by employing a short period of proteolytic digestion. The present isolation from the inner root sheath cells of morphologically distinct protein filaments which contain citrulline establishes the existence of this amino acid in the protein of the filaments and not primarily in a non-filamentous protein of these cells.

The constitution of the filaments in terms of the number of protein species that contain citrulline has not been determined. Such a deter-

mination will be difficult since recent work (H. W. J. Harding and G. E. Rogers, unpublished) has shown the presence of γ-peptide (isopeptide) links between adjacent polypeptide chains. These cross-links prevent the separation of the component chains and their dissolution by the usual solvents for proteins.

It has not been possible to establish a criterion of chemical purity for these inner root sheath filaments. Although the possibility of contamination of the filaments by other nonfibrous protein material cannot be overlooked, the amino acid analyses suggest that the extent of this contamination is minimal.

Despite the similar diameters of inner root sheath filaments and keratin microfibrils (approximately 80 Å), they differ from one another in at least two properties. Keratin microfibrils show a different affinity for the permanganate section-stain due to the presence of the sulfur-rich matrix within their cores and between their peripheries (11). Secondly, the amino acid composition of the inner root sheath filaments is entirely different from that of α-keratin. The virtual absence of cystine and the presence of citrulline are the salient features of difference.

*Possible functional relationship with microtubules.* The primary function of the inner root sheath is considered to be a structural one (12, 13). The cells of the sheath are thought to constrain the growing hair in the follicle and thus contribute a cooperative effect to the elongation of the cortical cells internal to them (12, 13). Further, the outward movement of the inner root sheath relative to the hair is regarded as being the cause of the flattened form of the cuticle cells by establishment of a "shearing" action (12). These morphogenic forces could be expected to be dependent upon the fibrous elements of the inner root sheath cell.

The structural elements that are prominently involved in the acquisition of cell shape during growth and development of many cells are the cytoplasmic microtubules; these structures are becoming well characterized especially in their occurrence as outer fibres of sperm tails (14) and cilia (15) and as microtubules in neurones (16–18). It is of interest that cytoplasmic microtubules are not prominent features of either developing or mature inner root sheath cells (unpublished observations of this laboratory). Thus it is suggested that the filaments of these cells replace microtubules as the determinants of cell shape and structure.

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